AVIAN DISEASES 48:647–650, 2004

Research Note—

Inability of Cecal Microflora to Promote Reversion of Viable Nonculturable *Campylobacter jejuni*

Richard L. Ziprin and Roger B. Harvey

Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, USDA, 2881 F & B Road, College Station, TX 77845

Received 5 January 2004; Accepted 20 April 2004

SUMMARY. *Campylobacter jejuni* cells are able to enter a viable but nonculturable (VBNC) state when they are suspended in water. In the present experiments we inoculated day-of-hatch leghorn and broiler chicks with normal gut microflora and subsequently challenged these with high doses of VBNC *C. jejuni*. The objective was to determine if the pre-establishment of a normal gut flora would enable VBNC *Campylobacter* to recover, revert to the vibrionic form, and colonize the cecum. Day-of-hatch leghorn and broiler chicks were gavaged through the esophagus with 0.75 ml of a continuous-flow culture of normal cecal organisms. Two days after gavage, the same chicks were gavaged with 0.75 ml (greater than 10^9 colony-forming units) of a VBNC suspension of *C. jejuni*. Seven days later, cecal contents were collected, serially diluted, and examined for the presence of viable culturable *C. jejuni*. Our results demonstrated that the VBNC *C. jejuni* cells were unable to revert to a vibrionic culturable form capable of colonizing the cecum.

RESUMEN. *Nota de Investigación*—Incapacidad de la microflora del ciego para promover la reversión de *Campylobacter jejuni* viable no cultivable.

Al ser suspendidas en agua, las células de *Campylobacter jejuni* son capaces de entrar en un estado viable no cultivable. Se inocularon al nacimiento pollitos tipo Leghorn y pollitos de engorde con una microflora intestinal normal y seguidamente fueron desafiados con una dosis elevada de *C. jejuni* viable no cultivable. Se determinó si el establecimiento inicial de una flora intestinal normal permitiría la reversión del *C. jejuni* viable no cultivable a su forma vibriónica, colonizando el ciego. Al nacimiento, se les introdujo a través del esófago 0.75 ml de un cultivo de organismos cecales normales por ave. A los dos días, a las mismas aves se les introdujo a través del esófago 0.75 ml de una suspensión de *C. jejuni* viable no cultivable (más de 10^9 unidades formadoras de colonia). Siete días después, se tomaron los contenidos cecales, se diluyeron seriadamente y se examinaron para determinar la presencia de *C. jejuni* viables cultivables. Se demostró la incapacidad de las células de *C. jejuni* viables no cultivables de revertir a su forma vibriónica cultivable capaz de colonizar el ciego.

Key words: *Campylobacter*, normal flora, viable nonculturable

Abbreviations: CFU = colony-forming units; doh = day of hatch; NAF = normal adult chicken microflora maintained in a continuous-flow chemostat; VBNC = viable but nonculturable

Rollins and Colwell (19) described a viable but nonculturable (VBNC) coccoid form of *Campylobacter jejuni* in natural aquatic environments. Nonculturable coccoid cells form when *C. jejuni* cells are suspended in water. Although these coccoid cells remain viable, they are undetectable by conventional microbiological methods. Instead, VBNC organisms may be detected by their ability to reduce tetrazolium salts (2,10) and, electron-microscopically, by the fact that the cells remain intact against high osmotic gradients.

Several bacterial genera contain organisms capable of entering the VBNC state (1,4,5,14,18,20,22,23). However, the physiological role of the VBNC state is somewhat unclear. Some evidence indicates that

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

VBNC cells are degenerative and destined to die (27,28,29). It has been reported that VBNC cells of *Salmonella typhimurium* do not infect mice, whereas it has been reported that VBNC *C. jejuni* do and do not colonize chickens (24,29). The questions surrounding formation of VBNC cells, possible resuscitation, infectivity after resuscitation, and conditions favorable to resuscitation of VBNC *C. jejuni* may be critical to understanding how poultry become infected within broiler houses. We do not yet know if either the morphologic variations or the VBNC state are part of *Campylobacter*'s approach to enhancing their opportunity to survive in the environment and their opportunity to infect animals and man (3,7,9,15,16,24,26,27,28,29).

Vibrio cholerae and *Helicobacter pylori* are pathogens of the human gastrointestinal tract that bear many similarities to *C. jejuni*. Each is, like *Campylobacter*, a vibrio with a polar flagellum, and each readily forms VBNC cells under adverse conditions. *Vibrio cholerae*'s natural habitat is aquatic, and it is in aquatic environments that *Campylobacter* transforms to the VBNC state. Colwell *et al.* (5) reported that human volunteers developed cholera following exposure to the VBNC form of the cells. That report (5) indicates that the human intestine, complete with normal adult microflora, presents an environment in which VBNC *V. cholerae* are resuscitated and transformed into infective cells. Given this fact, the objective of the present study was to determine if VBNC cells of *C. jejuni* would colonize leghorn chickens (layers), or broilers, that had previously been inoculated with microflora from chicken cecal contents.

MATERIALS AND METHODS

Animals. Day-of-hatch (doh) leghorn chickens (HyLine W36®) and Cobb × Ross broilers were obtained from local commercial hatcheries and placed in electrically heated commercial brooder batteries (10 chicks per cage). Feed was heat-treated at 65 C in an oven. Chlorinated municipal drinking water was provided in open troughs. Chicks were provided water and a balanced unmedicated corn-soybean ration *ad libitum.* An institutional Animal Care and Use Committee reviewed and approved husbandry and experimental procedures.

VBNC. A single *C. jejuni* strain, designated in our laboratory as *C. jejuni* 91, was used for each experiment. This *C. jejuni* 91 was originally isolated from bovine fecal matter and was subsequently found to be a capable colonizer of the ceca of doh chicks (30). Its ability to colonize chicks was verified prior to preparation of VBNC cells by challenging small groups of doh chicks (five chicks) at the onset of an experiment. Four campy-cefex agar plates (25) were heavily seeded with this organism in a manner that did not quite lead to confluent growth and were incubated for 48 hr at 42 C in a microaerophilic environment consisting of 5% oxygen, 10% carbon dioxide, and 85% nitrogen. These plates were then washed thoroughly with 5 ml of sterile water that had been purified by reverse osmosis. The resultant bacterial suspension was washed once by centrifugation, and the pellets were resuspended in 45 ml of sterile water. The suspension was allowed to stand at room temperature for 8 days, during which the cells transformed to the VBNC form, that is, the suspension did not contain culturable cells, but viable cells reduced tetrazolium (29). The VBNC cell concentration exceeded 10^9 particles, as estimated with the use of a Petroff Hauser bacterial cell counting chamber (29).

Continuous-flow cultures of normal gut flora. Normal adult chicken microflora (NAF) were obtained from a continuous-flow chemostat that has been maintained at our laboratory for many years. Details are described elsewhere (6,11,12,17). This culture is free from viable culturable *Campylobacter*.

Experimental design. Two essentially identical replicate experiments were conducted using layer chicks, and a third experiment was performed using a broiler breed. For each experiment, 50 doh chicks were placed, 10 per brooder battery, into five individual batteries. Birds in group 1 were untreated. Birds in group 2 were given, by gastric gavage, 0.75 ml of the water suspension of VBNC cells described above for doh. Birds in group 3 were given 0.75 ml NAF by gavage and received no further treatment. Birds in group 4 were treated as birds in group 3, with NAF on doh, but 2 days afterward were challenged by gavage with 0.75 ml of the VBNC cell suspension. This treatment with VBNC distinguishes group 4 from group 3. Birds in group 5 were given VBNC cells on the same day that birds in groups 4 were challenged with VBNC cells. One week after the birds in groups 4 and 5 were given VBNC cells, all birds were humanely euthanatized and their cecal contents were collected aseptically, serially diluted, and plated on campy-cefex agar plates to detect the presence of culturable *C. jejuni* in the ceca.

RESULTS AND DISCUSSION

Campylobacter jejuni were not recovered from any of the 150 chickens examined. The birds in group 1 served as a control against the possibility that chicks might become contaminated with *C. jejuni* either at the hatchery or through other accidental means while in our facility. The failure to recover *C. jejuni* from birds in treatment group 1 demonstrated that campylobacteria were not introduced from the

hatchery. The failure to recover *C. jejuni* from birds in group 2 demonstrated that no colonizing *C. jejuni* cells (e.g., residual vibrionic culturable cells) were present in the water suspension of VBNC cells. Neither did spontaneous change from the VBNC state to a colonizing form occur. Birds in group 3 served as a control against the presence of colonizing *Campylobacter* in the NAF culture and also served as a control against the accidental contamination of the poultry facility by campylobacteria. The failure to recover *Campylobacter* from birds in this group demonstrated that the NAF was free of *Campylobacter*.

The primary experimental group was group 4. Birds in this group received NAF 48 hr before they received VBNC. The absence of recoverable *C. jejuni* from birds in this group demonstrated the failure of NAF to create an intraluminal environment in which VBNC cells will revert to culturable colonizing forms. Hence, NAF did not create conditions in the cecum that would allow VBNC cells to become viable, culturable, colonizing cells.

Birds in group 5 controlled against the possibility that microflora acquired naturally within the poultry facility would induce transformation of VBNC cells to colonizing culturable cells. The failure to recover *C. jejuni* from this group demonstrated that naturally acquired microflora were incapable of creating an environment in which VBNC cells recovered their colonizing ability.

In our present experiment we gave doh chicks a bolus consisting of a mix of microflora that normally are present within the adult chicken cecum. We chose to use a continuous-flow culture derived from adult chicken cecal material instead of a suspension of normal adult chicken cecal contents because of the possible presence of *Campylobacter* in adult cecal material. Use of a continuous culture offers the advantage of a known *Campylobacter*-free culture. The disadvantage of choosing this approach lies primarily in the fact that the culture undoubtedly does not contain the full range of organisms, up to 500 species, present in the adult cecum.

Some authors have reported that commercial broiler flocks become contaminated and colonized with *Campylobacter* while in grow-out facilities (8,13,21,28). VBNC present in hatcheries, water, or in newly hatched chicks appears to be an attractive explanation for this contamination, as it can be argued that it takes time for the development of a normal gut microflora, which might be required for the transformation of VBNC cells to the replicating, viable, vibrionic form that is capable of colonization (28). But while this is an attractive theory, it is neither supported nor refuted by presently available information.

It is clear from the experiments with *V. cholerae*, conducted by Colwell *et al.* (5), that under the correct conditions, VBNC *V. cholerae* will revert to a culturable colonizing form. To date, our results, both in the present study and in a previously reported study (29), have failed to show a similar *C. jejuni* reversion back to the vibrionic colonizing form. Perhaps there are events or environmental conditions within the gastrointestinal tract that are necessary to enable change from the VBNC form. It is possible that VBNC cells are transiently capable of reversion to a colonizing form but that this transient status occurs early on, while there is still a mix of both vibrionic and coccoid cells (culturable and VBNC) in the water suspension. Finally, because NAF did not induce the VBNC cells to become viable, culturable, and colonizing, NAF obtained from a continuous-flow chemostat is safe for use as a competitive exclusion culture in situations in which chickens are exposed to VBNC cells.

REFERENCES

1. Boaretti, M., M. Del Mar Lleo, B. Bonato, C. Signoretto, and P. Canepari. Involvement of rpoS in the survival of *Escherichia coli* in the viable but non-culturable state. Environ. Microbiol. 5:986–996. 2003.

2. Cappelier, J. M., B. Lazaro, A. Rossero, A. Fernandez-Astorga, and M. Federighi. Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells. Vet. Res. 28: 547–555. 1997.

3. Chan, K. F., H. L. Tran, R. Y. Kanenaka, and S. Kathariou. Survival of clinical and poultry-derived isolates of *Campylobacter jejuni* at a low temperature (4 C). Appl. Environ. Microbiol. 67:4186–4191. 2001.

4. Colwell, R. R. Global climate and infectious disease: the cholera paradigm. Science 274:2025–2031.1996.

5. Colwell, R. R., P. Brayton, D. Herrington, B. Tall, A. Huq, and M. M. Levine. Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. World J. Microbiol. Biotechnol. 12:28–31. 1996.

6. Corrier, D. E., D. J. Nisbet, J. A. Byrd II, B. M. Hargis, N. K. Keith, M. Peterson, and J. R. Deloach. Dosage titration of a characterized competitive exclusion culture to inhibit *Salmonella* colonization in broiler chickens during growout. J. Food Prot. 61:796–801. 1998.

7. Federighi, M., J. L. Tholozan, J. M. Cappelier, J. P. Tissier, and J. L. Jouve. Evidence of non-coccoid viable but non-culturable *Campylobacter jejuni* in microcosm water by direct viable count, CTC-DAPI double staining,

and scanning electron microscopy. Food Microbiol. 15: 539–550. 1998.

8. Gregory, E., H. Barnhart, D. W. Dreesen, N. J. Stern, and J. L. Corn. Epidemiological study of *Campylobacter* spp. in broilers: source, time of colonization, and prevalence. Avian Dis. 41:890–898. 1997.

9. Hald, B., K. Knudsen, P. Lind, and M. Madsen. Study of the infectivity of saline-stored *Campylobacter jejuni* for day-old chicks. Appl. Environ. Microbiol. 67: 2388–2392. 2001.

10. Hatzinger, P. B., P. Palmer, R. L. Smith, C. T. Penarrieta, and T. Yoshinari. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. J. Microbiol. Methods 52:47–58. 2003.

11. Hume, M. E., A. G. Hollister, D. J. Nisbet, D. E. Corrier, and J. R. DeLoach. Effect of a characterized continuous-flow culture of cecal bacteria on *Salmonella typhimurium* crop colonization in broiler chicks. Avian Dis. 40:391–397. 1996.

12. Hume, M. E., D. J. Nisbet, and J. R. DeLoach. *In vitro* 14C-amino acid fermentation by CF3, a characterized continuous-flow competitive exclusion culture of caecal bacteria. J. Appl. Microbiol. 83:236–242. 1997.

13. Jacobs-Reitsma, W. F., A. W. Van de Giessen, N. M. Bolder, and R. W. A. Mulder. Epidemiology of *Campylobacter* spp at 2 Dutch broiler farms. Epidemiol. Infect. 114:413–421. 1995.

14. Johnston, M. D., and M. H. Brown. An investigation into the changed physiological state of *Vibrio* bacteria as a survival mechanism in response to cold temperatures and studies on their sensitivity to heating and freezing. J. Appl. Microbiol. 92:1066–1077. 2002.

15. Lazaro, B., J. Carcamo, A. Audicana, I. Perales, and A. Fernandez-Astorga. Viability of DNA maintenance in nonculturable spiral *Campylobacter jejuni* cells after long-term exposure to low temperatures. Appl. Environ. Microbiol. 65:4677–4681. 1999.

16. Medema, G. J., F. M. Schets, A. W. Van de Giessen, and A. H. Havelaar. Lack of colonization of 1 day old chicks by viable, non-culturable *Campylobacter jejuni*. J. Appl. Bacteriol. 72:512–516. 1992.

17. Nisbet, D. Defined competitive exclusion cultures in the prevention of enteropathogen colonisation in poultry and swine. Antonie Van Leeuwenhoek. 81: 481–486. 2002.

18. Pruzzo, C., R. Tarsi, M. Lleo Md Mdel, C. Signoretto, M. Zampini, R. R. Colwell, and P. Canepari. *In vitro* adhesion to human cells by viable but non-culturable *Enterococcus faecalis*. Curr. Microbiol. 45:105–110. 2002.

19. Rollins, D. M., and R. R. Colwell. Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52:531–538. 1986.

20. Saito, N., K. Konishi, F. Sato, M. Kato, H. Takeda, T. Sugiyama, and M. Asaka. Plural transformation-processes from spiral to coccoid *Helicobacter pylori* and its viability. J. Infect. 46:49–55. 2003.

21. Shane, S. M. *Campylobacter* infection of commercial poultry. Rev. Sci. Tech. Off. Int. Epizoot. 19:376–395. 2000.

22. Shleeva, M. O., K. Bagramyan, M. V. Telkov, G. V. Mukamolova, M. Young, D. B. Kell, and A. S. Kaprelyants. Formation and resuscitation of "non-culturable" cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. Microbiology 148:1581–1591. 2002.

23. Smith, R. J., A. T. Newton, C. R. Harwood, and M. R. Barer. Active but nonculturable cells of *Salmonella enterica* serovar *typhimurium* do not infect or colonize mice. Microbiology 148:2717–2726. 2002.

24. Stern, N. J., D. M. Jones, I. Wesley, and D. M. Rollins. Colonization of chicks by non-culturable *Campylobacter jejuni* spp. Lett. Appl. Microbiol. 18:333–336. 1994.

25. Stern, N. J., B. Wojton, and K. Kwiatek. A differential-selective medium and dry ice generated atmosphere for recovery of *Campylobacter jejuni*. J. Food Prot. 55:514–517. 1992.

26. Tholozan, J. L., J. M. Cappelier, J. P. Tissier, G. Delattre, and M. Federighi. Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. Appl. Environ. Microbiol. 65:1110–1116. 1999.

27. Van de Giessen, A. W., C. J. Heuvelman, T. Abee, and W. C. Hazeleger. Experimental studies on the infectivity of non-culturable forms of *Campylobacter* spp. in chicks and mice. Epidemiol. Infect. 117:463–470. 1996.

28. Ziprin, R. L. *Campylobacter* and Campylobacteriosis: what we wish we knew. In: Pre-harvest and post-harvest food safety: contemporary issues and future directions. R. C. Beier, S. D. Pillai, T. D. Phillips, and R. L. Ziprin, eds. Iowa State Press, Ames, IA. pp. 73–86. 2004.

29. Ziprin, R. L., R. E. Droleskey, M. E. Hume, and R. B. Harvey. Failure of viable nonculturable *Campylobacter jejuni* to colonize the cecum of newly hatched leghorn chicks. Avian Dis. 47:753–758. 2003.

30. Ziprin, R. L., C. L. Sheffield, M. E. Hume, D. L. J. Drinnon, and R. B. Harvey. Cecal colonization of chicks by bovine-derived strains of *Campylobacter*. Avian Dis. 47: 1429–1433. 2003.